THE TENTH C. L. OAKLEY LECTURE*

The success of plasmid-encoded resistance genes in clinical bacteria

An examination of plasmid-mediated ampicillin and trimethoprim resistance genes and their resistance mechanisms

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Introduction

Since the mid-1930s, clinically important bacteria have been subjected to successive onslaughts of antibacterial drugs. However, in response, the bacterial populations have overcome these attacks rapidly by the selection of resistant variants or by their ability to acquire novel genetic material. Indeed, the development of new antibacterial drugs has been dominated by the bacterium's ability to resist the previous generations of drugs. So it was that the use of penicillins proved so successful during the second World War when they were used against bacteria which had already become resistant to sulphonamides. However, this success was soon compromised when resistance to the early penicillins emerged in the wake of their excessive use. The development of resistance to these penicillins, coupled with their restricted spectrum, initiated a search for alternative antibacterial drugs. In a series of classic experiments, Anderson (1974) demonstrated that there was a close correlation between the clinical introduction of each new antibacterial drug and the emergence of bacteria resistant to it. It is probable, however, that not all antibacterial drugs have an equally devastating effect on the bacterial population and that some are more efficient in the selection of resistant variants than others.

In the past two decades, two groups of drug have been dominant in antibacterial drug usage in Britain and probably in the rest of the world; these are the α -aminobenzyl penicillins, ampicillin and amoxycillin, and the trimethoprim-based drugs, either trimethoprim used on its own or in combi-

nation with sulphamethoxazole (co-trimoxazole). In the treatment of urinary infections caused by enterobacteria, there has been a heavy reliance on these two groups of orally administered drugs. The two groups are equally effective in combating sensitive pathogens but behave very differently in their absorption from the gut. Unlike trimethoprim, ampicillin-based drugs are relatively poorly absorbed and significant levels of residual ampicillin penetrate the bacteria-rich areas of the bowel, providing a highly selective environment for resistant bacteria. Now, most resistance genes are carried on resistance plasmids (R-plasmids) which can carry several different resistance genes. Thus, if ampicillin is administered, the ampicillin-resistant strains which are selected are often resistant to trimethoprim as well. While studying the incidence of resistance to trimethoprim in Edinburgh, we found that there was an association between the emergence of trimethoprim resistance in gram-negative bacteria and previous treatment of the patient with an ampicillin-based antibiotic (Amyes *et al.*, 1981). All the trimethoprim-resistant bacteria isolated were found to contain R-plasmids which had linked genes conferring trimethoprim and ampicillin resistances. Thus, ampicillin usage was selecting plasmid-containing strains which were also trimethoprim resistant. There was no apparent association with the previous administration of trimethoprim-containing products as there was a much lower incidence of trimethoprim resistance (and ampicillin resistance) in clinical isolates from patients treated with trimethoprim-containing products (Amyes, 1983). If ampicillin usage really does select trimethoprim resistant bacteria, the reverse situation should also exist whereby a switch of policy from ampicillin to trimethoprim should result in a reduction in trimethoprim-resistant bacteria. In one Edinburgh hospital, the patients

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had been treated predominantly with ampicillin and the incidence of trimethoprim resistance was determined. A switch in prescribing policy was then made to the use of trimethoprim alone. During the following 5 months, the incidence of trimethoprim resistance fell despite the increased trimethoprim selection pressure (Amyes and Gould, 1984). Indeed, the incidence of trimethoprim resistance in Edinburgh has remained considerably lower than that of ampicillin resistance in gram-negative bacteria (Amyes *et al.*, 1986; Reid *et al.*, 1988a) despite the equivalent levels of drug usage (Amyes and Young, 1987).

Regardless of the manner in which the resistance has been selected, R-plasmid-mediated bacterial resistance to both trimethoprim and ampicillin has caused some major clinical problems. This review will concentrate on plasmid-mediated resistance to these two drugs.

Ampicillin resistance

Ampicillin resistance in clinical isolates of gram-negative bacteria became a problem after the introduction of ampicillin in 1962. It was soon recognised that the mechanism of resistance was by the production of β -lactamases, enzymes which hydrolyse the carbon-nitrogen bond of the β -lactam ring and render the molecule inactive. In gram-negative bacteria, the β -lactamase genes were found increasingly on bacterial plasmids which were able to transfer freely and to spread the gene rapidly through the bacterial population (Anderson and Datta, 1965). Biochemical examination of the enzymes has shown that, unlike those of the gram-positive bacteria, there appears to be a definite distinction between β -lactamases produced by the bacterial plasmid genes and those encoded by the bacterial chromosomes (Medeiros, 1988).

β -Lactamases have been classified by several different criteria. Richmond and Sykes (1973) divided all β -lactamases into five classes, on the basis of their substrate profiles and their susceptibility to a range of inhibitors. In this scheme, only two classes of enzyme (III and IV) encompassed the plasmid-encoded β -lactamases of gram-negative bacteria and they were distinguished from each other by their susceptibility to inhibition by cloxacillin. However, it was the separation of plasmid-encoded β -lactamases by analytical isoelectric focusing, and the ability to quantify the isoelectric point (pI), that revolutionised the classification of these enzymes (Matthew *et al.*, 1975). This system allowed very closely related β -lactamases to be distinguished and the enzymes could be

further typed by molecular mass (M_r) determinations and substrate profile (table I). Finer typing could be provided by the enzyme's susceptibility to known β -lactamase inhibitors.

Thirty-seven distinct plasmid-mediated β -lactamases have been identified (table I). There have been several surveys of plasmid-encoded β -lactamases, carried by β -lactam resistant gram-negative bacteria, to investigate their relative clinical importance (Simpson *et al.*, 1980, 1986; Roy *et al.*, 1983; Nandivada and Amyes, 1987; Reid *et al.*, 1988a). Most of these studies have concentrated on bacteria isolated in Europe, though one of them has examined the β -lactamases in clinical bacteria isolated in India (Nandivada and Amyes, 1987). Wherever the studies have been undertaken, the predominant plasmid-encoded β -lactamase has always been the same, TEM-1. The success of this β -lactamase may come from a combination of its rapid hydrolysis of many penicillins (table I) and the fact that its gene can be carried by a family of related transposons. The TEM-1 β -lactamase is rather more successful than the closely related TEM-2 enzyme, even though conventional biochemical tests are unable to distinguish between them (table I). The only difference between the two is the substitution of a lysine residue for glutamine at amino-acid position 39. However, the success of these two enzymes may soon be reversed. During the last year, five new plasmid-encoded TEM-related β -lactamases (TEM-3-TEM-7) have been found in Europe (table I) and they seem to have derived from the TEM-2 enzyme rather than TEM-1. The special properties of these enzymes allow them to hydrolyse the third-generation cephalosporins and confer some degree of resistance against them. This is a major success of the plasmid-encoded β -lactamases and augurs considerable problems for the clinical use of the new cephalosporins. However, in the general surveys, these β -lactamases have not yet emerged. Indeed, the other plasmid-encoded enzymes have been found only on very few or single occasions. The SHV-1 and OXA-2 β -lactamases have been found more often than the others but, even so, in a far smaller proportion than the TEM enzymes. In *Pseudomonas* spp., the most successful plasmid-encoded β -lactamase has been the PSE-4 enzyme (Williams *et al.*, 1984) but, until recently, it had been found only in *Pseudomonas* spp. and not in the enterobacteria.

In our Scottish survey of β -lactam resistance (Reid *et al.*, 1988a), the TEM-1 β -lactamase was responsible for the majority of the plasmid-encoded β -lactamases. However, a *Klebsiella* strain was

Table I. Biochemical properties of the plasmid-encoded β -lactamases in gram-negative bacteria

β-Lactamase	Relative rate of hydrolysis or Vmax*					M _r	pI	Transposon mediated	Reference
	AMP	CARB	OXA	CTX	CAZ				
<i>Broad-spectrum enzymes</i>									
TEM-1	106	10	5	<1		22 000	5.4	+	Hedges <i>et al.</i> (1974)
TEM-2	107	10	5	0		23 500	5.6		Hedges <i>et al.</i> (1974)
SHV-1	212	8	0	0		17 000	7.6	+	Petroceilou <i>et al.</i> (1977)
HMS-1	253	14	<2			21 000	5.2	+	Matthew <i>et al.</i> (1979)
TLE-1	67	13	4	6		19 800	5.55		Medeiros <i>et al.</i> (1985)
TLE-2	140	13				19 000	6.5		Reid <i>et al.</i> (1987)
ROB-1	186	25	6				8.1		Rubin <i>et al.</i> (1981)
LCR-1	145	4				44 000	6.5	+	Simpson <i>et al.</i> (1983)
NPS-1	223	18	40	<1		25 000	6.5		Livermore and Jones (1986)
BRO-1	103	78					5.6		Eliasson and Kamme (1985)
OHIO-1	140	11	<0.5			22 000	7.0		Shlaes <i>et al.</i> (1986)
CEP-2		48				36 200	8.1		Levesque <i>et al.</i> (1982)
SAR-2	101	48	64	20		36 000	8.3		Nandivada and Amyes (1987)
<i>Oxacillin-hydrolysing enzymes</i>									
OXA-1	382	30	197	22		23 300	7.4	+	Dale and Smith (1974)
OXA-2	179	15	646	1		44 600	7.45	+	Dale and Smith (1974)
OXA-3	178	10	336	0		41 200	7.1		Dale and Smith (1974)
OXA-4	438	39	220	63		23 000	7.5	+	Medeiros <i>et al.</i> (1985)
OXA-5	188	40	210	49		27 000	7.62	+	Medeiros <i>et al.</i> (1985)
OXA-6	596	46	1048	28		40 000	7.8	+	Medeiros <i>et al.</i> (1985)
OXA-7	545	48	702	31		25 000	7.65	+	Medeiros <i>et al.</i> (1985)
<i>Carbenicillin-hydrolysing enzymes</i>									
PSE-1 (CARB-2)	90	97	<2	27		28 500	5.7	+	Matthew and Sykes (1977)
PSE-2	267	121	317	16		12 400	6.1	+	Matthew (1978)
PSE-3	101	253		<1		12 000	6.9		Sawada <i>et al.</i> (1974)
PSE-4 (CARB-1)	88	150	8	1		32 000	5.3	+	Furth (1975)
CARB-3	100	147	12.5			31 000	5.75	+	Labia <i>et al.</i> (1981)
CARB-4	130	79	1			22 000	4.9	+	Philippon <i>et al.</i> (1986)
CARB-5							6.3		A. Philippon and G. Paul†
SAR-1	63	122	<0.1	<0.1		33 700	4.9		Reid and Amyes (1986)
AER-1	38	98	0.9	20		22 000	5.9	+	Hedges <i>et al.</i> (1985)
N-3	113	113				24 000	6.0		Takahashi <i>et al.</i> (1983)
N-29	124	128				22 000	6.9		Takahashi <i>et al.</i> (1983)
<i>Cephalosporin-hydrolysing enzymes</i>									
TEM-3 (CTX-1)	71	14.5		445	40	24 000	6.3		Labia <i>et al.</i> (1988)
TEM-4	50	12		300		24 000	5.9		Paul <i>et al.</i> (1987)
TEM-5	78	60		150	490		5.55		Petit <i>et al.</i> (1988)
TEM-6							5.8		Bauernfeind and Horl (1987)
TEM-7							5.4		Gutmann <i>et al.</i> (1985)
SHV-2	145		0	4		17 000	7.6	+	Kliebe <i>et al.</i> (1987)

AMP, ampicillin; CARB, carbenicillin; OXA, oxacillin; CTX, cefotaxime; CAZ, ceftazidime.

*The rate of hydrolysis for penicillin G = 100.

†Unpublished data.

isolated which possessed a 132-kb plasmid (pUK700) that encoded the production of a PSE-4 β -lactamase (Reid *et al.*, 1988b). Plasmid pUK700 was freely transferable to other enterobacterial species and to *P. aeruginosa*. When the plasmid was transferred to other members of the Enterobacteriaceae, the plasmid remained intact and was freely transferable onwards to other members of the same family. On the other hand, when transferred to *Pseudomonas*, the plasmid was lost but the gene remained intact. The expression of this gene in *P. aeruginosa* was much more efficient than it had been in the enterobacteria; the enzyme was produced in greater quantities and the enzyme kinetics showed that it was able to hydrolyse its substrates more efficiently. Thus, there had been a change in the enzyme's properties on the transfer of its genetic determinant from enterobacteria to *P. aeruginosa* and this seemed to be linked to a shift in the location of the gene from a plasmid to the bacterial chromosome. The shift was permanent and the gene could not be auto-transferred out of *P. aeruginosa*. However, the gene could be extracted into a standard *Escherichia coli* strain with the mobilising plasmid R18-18. In the new *E. coli* host, the gene was expressed as it had been in *P. aeruginosa*, not as it had been originally in the Enterobacteriaceae. This suggests that there is a permanent change in the gene as it enters *P. aeruginosa* and shifts its location to the bacterial chromosome, and raises the question as to the original host of the PSE-4 β -lactamase gene. We infer that it may have been the Enterobacteriaceae, even though it is now much more commonly found in *P. aeruginosa* and the enzyme is more efficiently expressed in this species (Reid *et al.*, 1988b).

Studies of β -lactam resistance in the developing countries have shown that ampicillin resistance is a much more serious problem than it is in Europe (Young *et al.*, in press). In Southern India, the incidence of ampicillin resistance was 84% amongst the Enterobacteriaceae and 76% in *E. coli* (Nandivada and Amyes, 1987). The TEM-1 β -lactamase was still the major plasmid-encoded enzyme. However, other plasmid-encoded β -lactamases previously recorded in the West were poorly represented. Amongst the *E. coli* plasmid-encoded enzymes, only TEM-2, OXA-2 and SHV-1 β -lactamases were found. On the other hand, there was a previously undiscovered plasmid-encoded enzyme, SAR-2 (Nandivada and Amyes, 1987). This β -lactamase has a very high iso-electric point but, unlike other enzymes of similar pI, it does not have preference for cephalosporins (table I). The biochemical properties of the SAR-2 β -lactamase

do not seem to give it any obvious superiority over other plasmid-encoded β -lactamases, though in the particular circumstances of antibiotic challenge in India this enzyme may confer a positive selective advantage.

Similarly in Africa, the TEM-1 β -lactamase has been shown to be the most successful β -lactamase and was, until recently, the only plasmid-encoded β -lactamase found in *Vibrio cholerae* strains (Rahal *et al.*, 1973; Hedges *et al.*, 1977; Threlfall *et al.*, 1980). Although oral rehydration is usually the recommended policy for cholera infection, various local reasons may dictate that antibiotic therapy is also used. In 1984, there was an epidemic of ampicillin-resistant *V. cholerae* biotype El Tor strains in Tanzania and the predominant β -lactamase was again the TEM-1 enzyme. However, two strains possessed the Inc C plasmids which encoded a novel β -lactamase with a very low pI (Reid and Amyes, 1986). This enzyme (SAR-1) had a high efficiency against penicillins but was not as effective against cephalosporins. In many tropical areas the use of cephalosporins is very low whereas there is high reliance on penicillins (Amyes and Young, 1987) and this may be reflected by the emergence of this enzyme. The SAR-1 enzyme is still the only β -lactamase other than TEM-1 found in *V. cholerae* strains, for no chromosomal enzyme has yet been found in this species.

Trimethoprim resistance

Trimethoprim was first introduced into general clinical use in the United Kingdom in 1968. It was first marketed at a time when ampicillin resistance was beginning to emerge and alternatives were being sought. It is a synthetic drug that had been shown to be active against the dihydrofolate reductases of bacteria and far less active against the equivalent enzyme in mammalian cells. The bacterial dihydrofolate reductase acts at a later stage in the same metabolic pathway as dihydropyrimidine synthetase, the enzyme that is inhibited by sulphonamides. For this reason, trimethoprim was not originally marketed as a single drug on its own, but was always administered with sulphonamides. A further rationale for this policy was that the use of two drugs concurrently would delay what was seen to be the inevitable emergence of trimethoprim resistant bacteria (Bushby and Hitchings, 1968). However, within 3 years of the clinical introduction of the trimethoprim-sulphamethoxazole combination (co-trimoxazole), bacteria conferring a high level of trimethoprim resistance emerged. Fleming *et al.* (1972) demonstrated that the trimethoprim

resistance gene was carried on R-plasmids. These first trimethoprim R-plasmids were all of the Inc W group (Hedges and Datta, 1972).

All previous plasmid-encoded resistance genes had manifested their resistance either by modifying the drug so that it was no longer active, like the β -lactamases, or by preventing the accumulation of the drug within the cell. The trimethoprim resistance mechanism was novel because the plasmid encoded an additional dihydrofolate reductase which, unlike the chromosomal enzyme, was insensitive to the action of trimethoprim. Thus the inhibited bacterial enzyme was by-passed by the plasmid-encoded dihydrofolate reductase (Amyes and Smith, 1974). The plasmid-encoded enzyme had quite different biochemical properties from any previously identified dihydrofolate reductase. It was much larger (M_r 35 000) and, although it was inhibited competitively by trimethoprim, effective inhibition required much higher concentrations of trimethoprim (Amyes and Smith, 1976). Trimethoprim resistance genes were soon found on plasmids from several incompatibility groups (Jobanputra and Datta, 1974) and two distinct groups of enzyme were recognised (Pattishall *et al.*, 1977). The type-I dihydrofolate reductase increased the minimum inhibitory concentration (MIC) of trimethoprim for the host strain from around 0.1 mg/L to >1000 mg/L. It was produced to a level 10 times higher than the chromosomal enzyme. The dihydrofolate

reductase was competitively inhibited by trimethoprim with a K_i of 7.4 μ M, which is about 1200 times higher than the K_i of the chromosomal enzyme (table II). This plasmid-encoded enzyme also conferred a high degree of resistance to the general dihydrofolate reductase inhibitor, methotrexate. Unlike the chromosomal enzyme, the type-I dihydrofolate reductase was heat labile. It was later shown to be made up of two identical subunits, each of 17.6 Kda, which are not active individually (Huovinen, 1987). On the other hand, the type-II dihydrofolate reductase was composed of four identical subunits, each of 9.5 Kda (Smith *et al.*, 1979; Zolg and Hänggi, 1981). Despite the fact that the two complete enzymes were of similar size, they were quite different from one another. Although the type-II enzyme is much more resistant to trimethoprim inhibition (K_i 6100 μ M), it still increases the MIC of trimethoprim for the host strain by approximately the same extent as the type-I enzyme. This may be a result of the lower level of the enzyme that is produced within the cell: it is usually produced at levels much lower than those of the chromosomal enzyme. The comparative levels of resistance produced by these two plasmid-encoded dihydrofolate reductases suggest that the resistance may be manifest by a combination of the inherent resistance of the enzyme and the quantity in which it is produced.

Other plasmid-encoded dihydrofolate reductases

Table II. Biochemical properties of the plasmid-encoded trimethoprim-resistant dihydrofolate reductases

Enzyme	Activity ratio*	Tp ID50 μ M	Mtx ID50 μ M	TD50 min.	DHF Km μ M	Tp Ki μ M	M_r	Countries where reported
<i>Plasmid enzymes</i>								
Type Ia	13.6	57	5.6	0.5	5.6	7.4	35 000	All continents
b	4.5	32	2.8	1.2	11	41	24 500	UK
Type IIa	0.85	70 000	1100	>12	4.6	6100	35 000	{ Europe, North & South America, Asia
b	0.23	80 000	750	>12	8.3	150	35 000	
c	0.07	20 000	1000	>12	4.2	400	34 000	
Type III	2.0	2.0	0.02	>12	0.4	0.15	17 000	New Zealand, USA, UK
Type IV	600	0.2	0.02	>12	37	0.063	46 700	India
Type V	76	23	3.5	3.0	15.5	3.2	5 000†	Sri Lanka
Type VI	68	465	95	0.4	118	162	10 000	South Africa
Type VII	10.6	30	3.0	1.5	20	7.0	11 500	UK
Type S1	35.8	50	0.002	>12	10.8	11.6	19 700	Australia, USA
<i>Chromosomal enzymes</i>								
<i>E. coli</i>	1.0	0.02	0.01	>12	1.2	0.006	21 000	—
<i>S. aureus</i>	13.4	0.04	0.002	>12	80.0	0.006	21 600	—

Tp—Trimethoprim; Mtx—methotrexate.

ID50—Dose of drug to give 50% inhibition of the enzyme.

TD50—Time in min at 45°C to give 50% inhibition of the enzyme.

* Activity ratio is the activity of enzyme related to the specific activity of the *E. coli* chromosomal enzyme.

† Apparent molecular weight determined by gel filtration.

were found that had properties very similar to those of the type-II enzyme but their different pIs in iso-electric focusing suggested that the enzymes were not identical (Broad and Smith, 1982). Their genes were sequenced and, although the amino-acid sequences of the enzymes were not identical, there was a high degree of homology (Stone and Smith, 1979; Zolg and Hänggi, 1981; Flensburg and Steen, 1986). Thus the type-II enzymes can be divided into three groups; the majority of enzymes falling in group a, the prototype being encoded by plasmid R67, and a smaller number of enzymes represented in group b, the prototype for this gene being carried on plasmid R388. Only the dihydrofolate reductase from plasmid R751 falls into category c. Although the gene for this enzyme has been reported on a transposon and it was originally found on a promiscuous Inc P plasmid (Shapiro and Sporn, 1977), it has not been found generally within the population of clinical isolates.

In the surveys of clinical enterobacterial strains isolated in Edinburgh, the percentage of strains resistant to trimethoprim (MIC > 10 mg/L) did not increase significantly (Amyes *et al.*, 1978, 1981). However, the proportion of these bacteria that were highly resistant to trimethoprim (MIC > 1000 mg/L) increased much more rapidly. Before 1981, almost every highly resistant organism possessed a transferable trimethoprim R-plasmid. When the trimethoprim-resistance mechanism was examined, it was found to result invariably from either a type-I or type-II enzyme. In particular, the type-I dihydrofolate reductase predominated. Datta's group showed that the gene encoding this enzyme was located on a transposon, *Tn7*, which could move from one plasmid to another (Barth *et al.*, 1976). Despite the ubiquitous nature of *Tn7* and its obvious success in spreading through the bacterial population, it was unlike the promiscuous TEM-1 β -lactamase transposons because, for a long time, it remained exclusively within bacteria of the Enterobacteriaceae. This situation changed in 1984, when the *V. cholerae* biotype E1 Tor strains responsible for the cholera epidemic in Tanzania acquired a 113-kb resistance plasmid capable of conferring high-level trimethoprim resistance. The properties of the dihydrofolate reductase encoded by this plasmid indicated that it was the same type-I enzyme found in enterobacterial plasmids (Young and Amyes, 1986a).

By 1981, the proportion of trimethoprim resistant (MIC > 10 mg/L) strains in general hospitals in Edinburgh was 21% amongst the Enterobacteriaceae. About a quarter of these strains were highly resistant to trimethoprim and all these highly

resistant strains could transfer the trimethoprim-resistance gene (Amyes *et al.*, 1981; Amyes, 1983). Studies in one specialised hospital, however, showed a very different pattern. There was a much higher incidence (64%) of trimethoprim resistance and nearly half of the resistant strains were able to overcome trimethoprim at 1000 mg/L (Young and Amyes, 1983). Unlike the isolates from the general hospitals, not all these highly resistant strains were able to transfer their trimethoprim resistance. Indeed, close examination of the strains revealed that many of these strains possessed no plasmid DNA at all. This heralded a fundamental change in the location of the trimethoprim resistance genes as they were migrating from the exclusive carriage on bacterial plasmids on to the bacterial chromosome. This trend was later observed in the general hospitals in Edinburgh where, by 1984, the proportion of highly resistant strains carrying trimethoprim R-plasmids had fallen to 5.1% (Amyes *et al.*, 1986). The vast majority of the trimethoprim-resistance genes encoded the type-I dihydrofolate reductase, therefore the migration was the result of transposition of *Tn7* into the bacterial chromosome (Amyes *et al.*, 1986). It looked as though the migration of the resistance gene into the chromosome was going to be a continuing trend. However, the results of the latest study in 1986 suggest that the trend may now have been reversed and more trimethoprim R-plasmids exist within the population (Young and Amyes, 1988).

In the specialised hospital in Edinburgh, some strains carried plasmids which possessed a smaller transposon, *Tn4132*, that contained only a trimethoprim-resistance gene and not the streptomycin-resistance gene associated with *Tn7*. The dihydrofolate reductase of this transposon had very similar properties to the type-I enzyme (table II) (Young and Amyes, 1985), but it was smaller in size (M_r 24 500). The gene for this dihydrofolate reductase hybridises with a gene probe that is specific for the type-I gene (K. J. Towner, personal communication). Thus, the new enzyme was related to the original type I. Therefore, we have classified it as type Ib and reclassified the type I as type Ia (Amyes, 1986).

For many years, only the type-I and -II dihydrofolate reductases were identified from trimethoprim-resistant isolates found throughout the world. They were always indicated by the high MIC of trimethoprim that they conferred on the host cell. Fling *et al.* (1982) identified transferable trimethoprim resistance in a *Salmonella* strain isolated in New Zealand. This strain was distinguishable from strains producing the type-I or -II enzymes because

the plasmid (pAZ1) conferred a relatively low level of resistance (MIC 64 mg/L). The biochemical properties of the dihydrofolate reductase produced by the plasmid (Fling *et al.*, 1982; Joyner *et al.*, 1984) and the subsequent determination of the nucleotide sequence of the gene (Fling *et al.*, 1988) revealed that it was quite different from the two previous types. The type-III enzyme was considered to be a freak for many years because it had been found only on that one occasion. Recently, however, a type-III-like resistance gene has been found in plasmids carried by *Shigella* spp. responsible for two separate epidemics in the southern USA (Barg *et al.*, 1988). The identification of this resistance gene in enteric bacteria suggested that, as a resistance mechanism, the type-III enzyme would be confined to bacteria that would not experience high levels of trimethoprim and this would explain its rarity. However, this year (1988) we have found the type-III resistance gene in *E. coli* responsible for urinary infection in the UK and there is further evidence that the gene may be carried on a transposon (K. J. Towner, C. J. Thompson, H-K. Young and S. G. B. Amyes, unpublished results). If it is carried on a transposon, its "rarity" may derive from a lack of its detection rather than its scarcity. Most laboratories looking for transferable trimethoprim resistance usually examine only those strains with a high MIC of trimethoprim.

In a field study at the Christian College Medical College in Vellore, Southern India, we found that the incidence of trimethoprim resistance was very much higher amongst urinary pathogens (64%) than it had been in general hospitals in the UK (Young *et al.*, 1986a). About 90% of these resistant strains were highly resistant (MIC > 1000 mg/L), indicating a plasmid origin for the resistance genes. Indeed, a higher proportion of highly resistant strains in India did carry trimethoprim R-plasmids than had been found in the UK (Young *et al.*, 1986a). Where possible, all trimethoprim-resistance genes were transferred to standard *E. coli* strains either by conjugation or mobilisation. There were 12 trimethoprim-resistant transconjugant strains which behaved differently from the rest. Unlike the findings with strains harbouring the types-I-III dihydrofolate reductases, the MIC of trimethoprim for the different strains was very dependent on the testing medium (Young *et al.*, 1986b). In the 12 strains there were seven related plasmids which produced a novel dihydrofolate reductase that had biochemical properties quite distinct from those of the types-I-III enzymes (Young and Amyes, 1986b). This type-IV dihydrofolate reductase gave less protection against trimethoprim inhibition than

the others and no protection against the 2,4-diamino-pteridine, methotrexate (table II). However, a strain which produces the type-IV dihydrofolate reductase is able to overcome moderately high levels of the drug. The type-IV enzyme is inducible and the level of dihydrofolate produced is in direct proportion to the challenge dose of trimethoprim (Young and Amyes, 1986b). It is the only plasmid-encoded trimethoprim-resistance gene that is inducible and is one of only two or three plasmid-encoded resistance genes in gram-negative bacteria that are. Thus, even more so than the type-I or -II enzymes, the mechanism of resistance of the type-IV gene results from both the production of a dihydrofolate reductase that is less sensitive than the bacterial enzyme, and the increased synthesis of this enzyme at higher drug challenge. As yet, the type-IV dihydrofolate reductase has been found nowhere other than Southern India.

In studies of transferable trimethoprim resistance in Sri Lanka, highly resistant strains were found which possessed R-plasmids that did not hybridise with the type-I or -II gene probes (Sundstrom *et al.*, 1987). The plasmid encoded a dihydrofolate reductase which preliminary inhibition data suggested was very similar to the type-I enzymes. Sköld (1988) sequenced the gene encoding this type-V enzyme and predicted a protein of 157 amino acids and a size of 18 Kda. There are considerable similarities between the amino-acid sequence of the type-V and the type-Ia dihydrofolate reductases (Thomson and Amyes, 1988). Further biochemical analysis of the type-V enzyme suggested that it is virtually indistinguishable from the type-Ia or -Ib enzymes (table II). However, gel filtration revealed that the size of the type-V protein was 5 Kda (Thomson and Amyes, 1988). The existence of the type-Ib dihydrofolate reductase in clinical bacteria suggests that it is the N-terminal region of the protein that is responsible for the active site and the enzymic action. Thus another type-I-like enzyme might indeed be small and fully functional.

There are other areas of the world where transferable high-level trimethoprim resistance genes do not hybridise with the types-I or -II gene probes. In South Africa, a considerable proportion of plasmid-derived trimethoprim resistance genes do not hybridise with the types-I or -II gene probes. A *Proteus mirabilis* strain was examined in more detail. It carried a 79-kb plasmid (pUK672) which was freely transferable into standard *E. coli* strains. The biochemical properties of this plasmid-encoded enzyme (type VI) showed that it was smaller (M_r 10 000) than all the other trimethoprim-resistant dihydrofolate reductases except the type-V

enzyme (Wylie *et al.*, 1988). On the other hand, it was more resistant to both trimethoprim and methotrexate inhibition than all except the type-II enzymes. In fact, the inhibition properties of the type-VI dihydrofolate reductase were approximately mid-way between the types I and II (table II) (Wylie *et al.*, 1988). The M_r of the enzyme and its inhibition properties suggest that it may derive from a sub-unit of one of the type-II dihydrofolate reductases, even though the sub-units of the type-II enzymes are themselves inactive. The type-VI enzyme has, so far, been found only in South African strains.

Very recently, some highly resistant strains, which are capable of transferring trimethoprim resistance and which do not hybridise with the types-I-V gene probes, have been found in clinical isolates in the UK. Examination of these strains has revealed the presence of a new dihydrofolate reductase with similar properties to the types-I and -V enzymes (table II). However, the very distinctive size of the enzyme (M_r 11 500) and the inability of its gene to hybridise with the type-I and -V gene probes have placed it in a new group (type VII) (S. G. B. Amyes, H-K. Young and K. J. Towner, unpublished results).

Trimethoprim resistance in gram-positive bacteria has been slower to develop and it was not until the emergence of multiresistant *Staphylococcus aureus* that transferable trimethoprim resistance was found. Trimethoprim R-plasmids were found in *S. aureus* strains isolated in Australia and the USA (Lyon *et al.*, 1983, 1984; Archer *et al.*, 1986). The trimethoprim resistance gene from the original Australian trimethoprim R-plasmid (pSK1) was cloned into the multicopy vector plasmid pACYC184 and the trimethoprim resistance mechanism examined in an *E. coli* strain harbouring the vector plasmid pSK407. The staphylococcal trimethoprim resistance gene (*dfpA*) encoded a novel trimethoprim-resistant dihydrofolate reductase (type S1) (Young *et al.*, 1987). This enzyme gave the same level of protection to trimethoprim inhibition as the type-I enzymes of gram-negative bacteria. However, its other biochemical properties were quite different (table II). It was smaller (M_r 19 700) than the type-I enzyme; it was heat resistant and gave no protection against methotrex-

ate inhibition. It seems similar to the plasmid-encoded enzyme subsequently discovered in *S. aureus* strains isolated in the USA (Coughter *et al.*, 1987). We have also found the type-S1 enzyme encoded by different plasmids in *S. epidermidis* and *S. hominis* as well as *S. aureus* (Tennent *et al.*, 1988) and by the chromosome of a clinical isolate of *S. epidermidis*.

Conclusions

In recent years, there has been a host of discoveries of new plasmid-encoded β -lactamases and dihydrofolate reductases. In the case of the β -lactamases, this has resulted from the examination of clinical strains from new areas and the development of resistance to third-generation cephalosporins. With the dihydrofolate reductases, the increasing frequency with which new enzymes are being found comes from the use of specific gene probes and, possibly also, from discarding the old preconceptions that all plasmid-mediated enzymes must be either type I or type II. However, despite the myriad of different enzymes for ampicillin and trimethoprim resistance, an evolutionary pattern has arisen. In the case of the β -lactamases, the enzymes can be classified into distinct groups (table I), in which there are considerable biochemical similarities. The majority of enzymes in the largest and most common group, the broad-spectrum β -lactamases, have very similar biochemical properties. The two most common enzymes, TEM-1 and TEM-2, are very closely related to the new cephalosporin-hydrolysing enzymes. Similarly, many of the dihydrofolate reductases found have properties comparable to those of the type-Ia enzyme (table II), suggesting that there is some evolutionary advantage for this combination.

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